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FERMENTATION KINETICS OF BOTULINUM TOXIN PRODUCTION (TYPES A, B AND E)

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INTRODUCTION

The botulinum toxoid currently in use for human immunization is derived from formalin-inactivated types A, B, C, D and E toxins. It was produced in 1958 by Parke-Davis, under contract to the United States Army. For type A, the preparation contains only about 10% neurotoxoid (1). Similar low percentages of purity are to be expected for the other types. This toxoid elicits sustained, measurable antibody titers only after a series of four injections over a period of one year (2). Mild side-reactions are common, including itching, tenderness, redness and swelling at the site of injection (3). A new product is required: one that includes types F and G, and that is prepared from highly purified neurotoxins. This is the goal of this research effort. To produce such a product, suitable for human immunization against types A-G, methods must be developed to: (a) produce each toxin in large quantities, (b) purify the neurotoxin from the culture fluid, and (c) convert each neurotoxin into a neurotoxoid and combine them into a polyvalent product.

To produce botulinum toxin in large quantities, previous workers have used cultures grown statically in carboys. The problems inherent in the use of carboys as growth vessels for the production of clostridial vaccines are many, and have been emphasized by Hepple (4). In contrast, we are employing a fermentor for botulinum toxin production. The numerous advantages of using a fermentor system for the cultivation of anaerobic bacteria have been noted by Sargeant (5). A fermentor allows for the precise, continuous measurement and control of

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temperature, pH, redox potential and the rate of agitation and sparging. Cultures in stirred fermentors are homogeneous and representative samples can easily be obtained without risk of aeration. In addition, the use of a fermentor facilitates the correlation of the appearance of the desired product with specific phases of growth. Thus, the aims of our fermentor studies are to determine the optimal conditions for toxin production (with respect to the initial concentration of growth medium components, pH, gas flow, agitation and temperature) and the relationship between bacterial growth and toxin production.

Studies have been conducted using a 70-liter fermentor containing 50 liters of medium. The medium consists of casein hydrolysate, yeast extract, plus an appropriate concentration of glucose. A complex medium is used, since such media have been reported to support approximately 10-fold more toxin production than do chemically defined media (6). This medium was chosen because of its suitability for toxin production and its economic feasibility for large-scale use.

CLOSTRIDIUM BOTULINUM TYPE A

We first examined the growth of C. botulinum type A, Hall strain, and the appearance of toxin in the culture fluid over a time course of 72 hours (Fig. 1). As measured by optical density, growth was exponential for about 6 hours (mean generation time, 76 min). The oxidation-reduction potential (Eh), recorded at the pH of the culture, declined from the time of inoculation to reach a minimum at 5 hours and then rose during the course of the experiment. The pH, which was not regulated, decreased to 5.4 by 24 hours and did not increase from that value for the remainder of the 72 hours.

The amount of toxin in the culture fluid increased during the first 24 hours to a maximum of 6.3×10^5 mouse intraperitoneal LD_{50}/ml. Toxin accumulation was not augmented by continued incubation. When the growth and the toxin curves are examined together, it is evident that the toxin titers rose during the logarithmic and stationary phases of growth, and that the maximum concentration of toxin was attained before the cells had lysed to an appreciable extent. Although previous investigators have maintained that autolysis is the mechanism by which toxin is liberated from C. botulinum type A (7, 8), cell lysis is apparently not required to obtain maximum toxin concentrations in the culture fluid when this organism is grown under the fermentation conditions described.

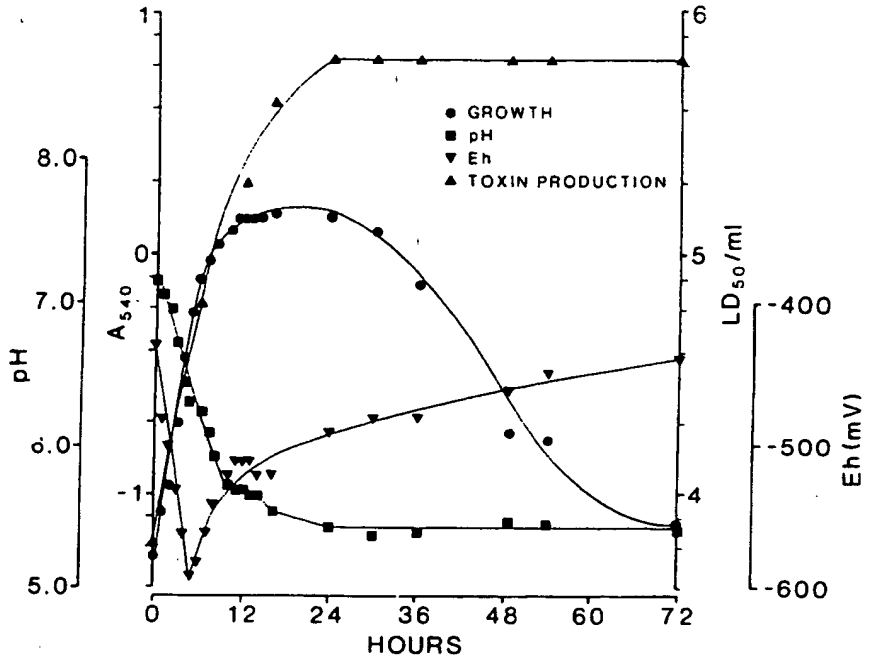

FIGURE 1. Growth, toxin production and cultural conditions of C. botulinum type A, Hall strain. The medium consisted of 2.0% casein hydrolysate and 1.0% yeast extract adjusted to pH 7.3, plus 0.5% glucose. Fermentation was at 35°C with an agitation rate of 50 rpm and a nitrogen overlay (5 liters/min).

In many fermentations by anaerobes, product yields have been increased by changing the temperature, pH or some other growth condition (5). The goal in such efforts is to decrease the rate of cell growth and thus to extend the production time. Therefore, the effects of the rate of agitation, together with the rate of nitrogen sparging of the culture versus a nitrogen overlay, on growth and toxin production were determined (9). Maximum growth was obtained in 16 hours in all studies, with lysis essentially complete at 72 hours. Using a nitrogen overlay (5 liters/min) with an agitation rate of 50 rpm, the maximum toxin concentration (6.3×10^5 LD_{50}/ml) was attained within 24 hours. Continued fermentation for up to 126 hours did not augment toxin accumulation. With nitrogen sparging at 5 liters/min and agitation at 50 rpm or at 10 liters/min and agitation at 100 rpm, toxin appearance was delayed, and titers subsequently decreased on continued incubation. Data obtained with CO_2 sparging (1 liter/min) were similar to those with nitrogen sparging at 5 liters/min.

Fermentations were conducted with varying concentrations of glucose: 1.5, 1.0, 0.5 and 0.25%, as well as no added carbohydrate (9). Growth was dependent on glucose concentration up

to 1.0%, but significant lysis occurred only in the presence of 0.5%. With 1.0 and 1.5% glucose, the maximum toxin concentration was attained in 24 hours; with 0.5% glucose, it was attained in 30 hours. Cultures supplemented with 0.25% glucose and those to which no carbohydrate was added produced much less toxin.

The addition of carbohydrate during growth has been reported to increase product yield (4). Therefore, after 8 hours of growth in a medium initially supplemented with 1.0% glucose, an addition of glucose was made to the culture to yield a further 1.0% glucose. However, this procedure did not increase toxin concentrations beyond those obtained with 1.0% glucose only (9).

The effect of temperature in the range from 30 to 45°C on growth and toxin production was examined (9). Growth occurred at all temperatures tested, but 40°C was optimal. However, of the temperatures tested, the optimum for toxin production was 35°C, with maximum titers produced in 24 hours. Toxin production was markedly reduced when an incubation temperature of 45°C was used.

Product yield has been reported to be increased by controlling the pH of the culture (4, 5). For type A, the pH (which was 7.1 after inoculation) was uncontrolled until pH 6.0 was reached, which occurred after approximately 8 hours of growth. The pH was then maintained at 6.0 for the duration of the experiment. The growth rate was unaffected by pH control, and the concentration of toxin was not increased by this procedure (9).

Thus, using a fermentor system, optimal production of C. botulinum type A toxin occurs at 35°C, with an agitation rate of 50 rpm and a nitrogen overlay of 5 liters/min, with an initial glucose concentration of 1.0%, in the absence of pH control. Under these conditions, maximum yields of toxin (6.3×10^5 LD_{50}/ml) are attained within 24 hours. In contrast, using static cultures requires 4 to 5 days incubation to obtain comparable toxin concentrations (10-13).

C. BOTULINUM TYPE B

Analogous studies have been performed with the bean strain of type B (14). The time course of growth and toxin production is shown in Figure 2. Growth was exponential for about 5 hours (mean generation time, 64 min), with maximum growth obtained in 12 hours. Lysis then occurred, followed by a resumption of growth, and a long stationary phase. The Eh decreased from the time of inoculation until 5 hours, remained

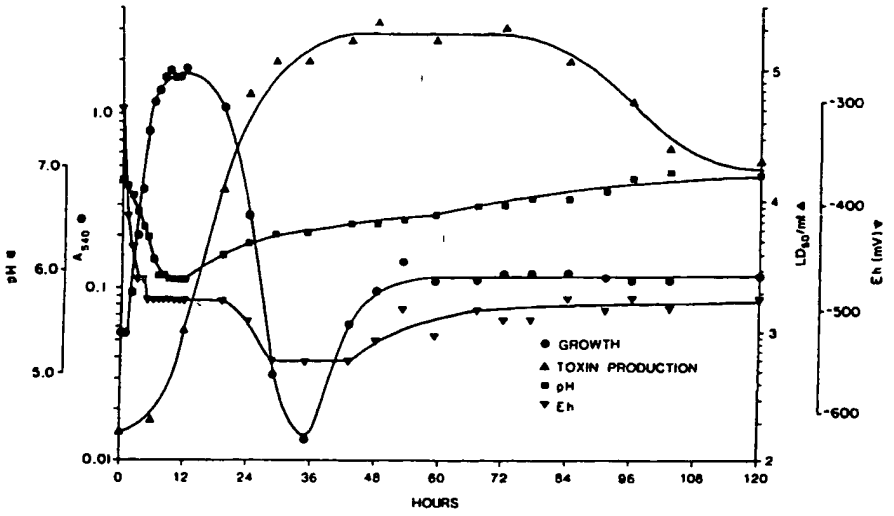

FIGURE 2. Growth, toxin production and cultural conditions of C. botulinum type B, bean strain. The medium consisted of 2.0% casein hydrolysate and 1.5% yeast extract adjusted to pH 7.0, plus 0.5% glucose. Fermentation was at 35°C with an agitation rate of 50 rpm and a nitrogen overlay (5 liters/min).

constant until 19 hours, then declined to a minimum at 29 hours, and remained at this value until 43 hours. It then increased over the remainder of the time period studied. The pH decreased to 5.9 at 9 hours, but after 12 hours, increased gradually for the duration of the run.

The toxin concentration in the culture fluid rose during the first 48 hours to a maximum of 2.5×10^5 LD_{50}/ml. Toxin titers were not increased by continued incubation, but declined with time after 72 hours. When the growth and toxin curves are examined together, it is clear that the maximum toxin concentration was attained only after lysis of the cells had occurred.

The effects of a nitrogen overlay versus sparging with nitrogen or with CO_2 were determined. In all studies, maximum growth was obtained in 12 hours. With the nitrogen overlay (5 liters/min), the maximum toxin concentration (4×10^5 LD_{50}/ml) was obtained in 48 hours. Less toxin was produced when the culture was sparged with nitrogen (5 liters/min) or with CO_2 (1 liter/min).

The effects of glucose concentration on growth and toxin production were determined. Significant lysis of the culture occurred with 0.25, 0.5 and 1.0% glucose, but increasing the glucose concentration delayed the time at which lysis of the culture began. In cultures supplemented with 0.5 and 1.0% glucose, maximum toxin titers were attained in 48 hours. Less

toxin was produced with 0.25 and 1.5% glucose, and only low toxin concentrations were obtained in the absence of added carbohydrate.

The effects of temperature on growth and toxin production were examined in the range from 25 to 40°C. A temperature of 40°C was optimal for growth, but growth occurred at all temperatures tested. However, the optimum for toxin production was 35°C, with maximum concentrations produced in 48 hours. Incubation at 25 or 40°C reduced toxin production.

Thus, the conditions required by the bean strain of type B for maximum toxin production in the fermentor system (14) are identical to those determined for the Hall strain of type A (9). However, the highest toxin yields were obtained in 24 hours for type A (Hall), but 48 hours of incubation were required to attain the maximum toxin concentration for type B. In contrast, incubation times of from 2 to 3 days, up to 10 days, are reportedly necessary to obtain comparable yields of type B toxin in static cultures (15-18).

C. BOTULINUM TYPE E

The growth and toxin production of C. botulinum type E, strain E43, were examined over a time course of 96 hours in the fermentor system (Fig. 3). The mean generation time during exponential growth was 62 min. Lysis of the culture did not occur. The Eh decreased from the time of inoculation to reach a minimum at 9 hours, remained at that value until 12 hours, then increased gradually over the remainder of the time period. The pH of the culture fluid decreased to 4.8 at 24 hours and remained at that value for the duration of the run. At the times indicated, samples of the whole culture were treated with trypsin (100 µg/ml at 35°C for 60 min), diluted serially and injected into mice to determine toxin concentration. The maximum amount of trypsin-activated toxin (2.0×10^5 LD_{50}/ml) was produced after 12 hours of fermentation. Continued fermentation did not increase toxin concentration.

For type E, strain E43, fermentation at 30°C with an agitation rate of 50 rpm and a nitrogen overlay at 5 liters/min yields a toxin concentration of 2.0×10^5 LD_{50}/ml (trypsin-activated) in 12 hours.

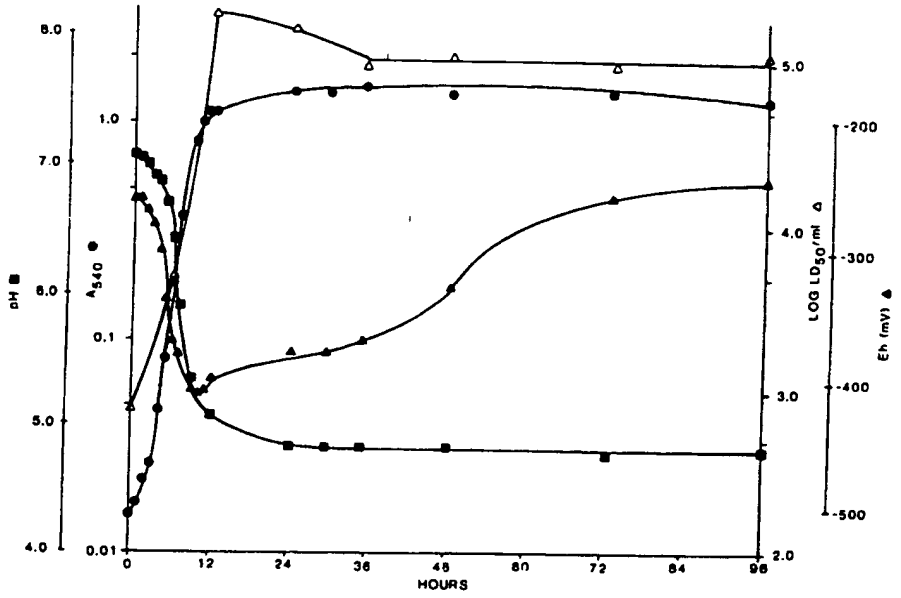

FIGURE 3. Growth, toxin production and cultural conditions of C. botulinum type E, strain E43. The medium consisted of 2.0% casein hydrolysate and 0.5% yeast extract adjusted to pH 7.2, plus 1.0% glucose. Fermentation was at 30°C with an agitation rate of 50 rpm and a nitrogen overlay (5 liters/min).

CONCLUSIONS

Thus, for types A, B and E C. botulinum, the concentrations of toxin produced using the fermentor are at least as high as those obtained with carboys. However, with the fermentor, toxin can be produced in a shorter period of time. In addition, the toxin concentrations obtained are reproducible from one batch to the next, using the same conditions.

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In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory

Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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